Insertion sequence *ISEcp1*-like element connected with a novel *aph(2'')* allele [*aph(2'')-Ie*] conferring high-level gentamicin resistance and a novel streptomycin adenyltransferase gene in *Enterococcus*

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Enterococcus casseliflavus HZ95 is an enterococcus with high-level resistance to aminoglycosides. Nine genes responsible for high-level aminoglycoside resistance, including *aac(6')-Ie-aph(2'')-Ia*, *aph(2'')-Ib*, *aph(2'')-Ic*, *aph(2'')-Id*, *aph(3'')-IIIa*, *aac(6'')-II*, *ant(3'')-Ia*, *ant(4'')-Ia* and *ant(6'')-Ia*, were not detected in HZ95. An 8 kb fragment from unconjugative plasmids of HZ95 was cloned, and expressed gentamicin resistance in *Escherichia coli* DH5 α . The genetic structures (~ 8 kb DNA fragment) containing these aminoglycoside-modifying enzyme genes in *Ent. casseliflavus* HZ95 were determined. The deduced amino acid sequence of the novel *aph(2'')* allele, *aph(2'')-Ie*, had 93.7% amino acid identity with APH(2'')-Id. The *aph(2'')-Ie* gene was bracketed upstream by an insertion sequence (IS)*Ecp1*-like element and downstream by a streptomycin adenyltransferase gene (*str*). The streptomycin adenyltransferase encoded by the *str* gene had 80.3% amino acid identity with the protein encoded by *aadE*. The plasmid of ~ 16 kb could hybridize with a PCR-generated *aph(2'')-Ie* intragenic probe. The *ISEcp1*-like element had 91% identity with *ISEcp1*. *ISEcp1*, which commonly acts as a key factor in the dissemination of CTX-M-type β -lactamase genes in Gram-negative bacteria, has not been reported in *Enterococcus*.

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INTRODUCTION

The incidence of nosocomial enterococcal infections has increased, and enterococci have become one of the most common nosocomial pathogens (Schaberg *et al.*, 1991; Murray, 1998). Optimal therapy of serious enterococcal infections usually involves a synergistic combination of an agent that interferes with cell wall biosynthesis, such as ampicillin, and an aminoglycoside. Gentamicin has been the preferred aminoglycoside; however, the increasing prevalence of high-level gentamicin resistance (HLGR) (as high as 52.6% in China) (Wang *et al.*, 1999), which eliminates this synergistic bactericidal effect, has compromised the effectiveness of therapy. The *aac(6')-Ie-aph(2'')-Ia* gene is responsible for the HLGR of strains by encoding AAC(6')-Ie-APH(2'')-Ia, a gentamicin-modifying enzyme

(Ferretti *et al.*, 1986). This gene for a bifunctional enzyme confers resistance to essentially all clinically available aminoglycosides, except streptomycin, and eliminates the synergism between aminoglycosides and cell-wall-active agents. Three novel genes encoding phosphotransferases, including APH(2'')-Ic, APH(2'')-Id and APH(2'')-Ib, have been detected (Chow *et al.*, 1997; Tai *et al.*, 1998; Kao *et al.*, 2000). The detection of resistance genes is employed to predict bactericidal synergism in enterococci.

Resistance to streptomycin can be mediated by ribosomal mutation or by the enzymic modification of the drug. Ribosomal resistance is usually associated with streptomycin MICs $> 32\,000\ \mu\text{g ml}^{-1}$, while enzyme-mediated resistance produces lower MICs (Eliopoulos *et al.*, 1984). The *aadE* gene (also referred to as the 6'-*ant* gene) is the main streptomycin adenyltransferase gene detected in *Enterococcus* (Werner *et al.*, 2001; Derbise *et al.*, 1997). The *aadA* gene (also referred to as the 3'-*ant* gene) has also been reported in Gram-negative organisms and *Staphylococcus aureus*, but has not been reported in *Enterococcus* (Ahmed & Shimamoto, 2004; Clark *et al.*, 1999).

Abbreviations: AME, aminoglycoside-modifying enzyme; HLGR, high-level gentamicin resistance; IS, insertion sequence.

The GenBank/EMBL/DDBJ accession number for the cloned fragment sequence of *Ent. casseliflavus* HZ95 containing the novel HLGR gene *aph(2'')-Ie* is AY939911.

Insertion sequences (ISs) are an important source of genetic plasticity in prokaryotes. Tn5281 is often associated with the dissemination of the *aac(6')-Ie-aph(2'')-Ia* gene with IS256 upstream and reverse IS256 downstream in HLGR strains (Simjee *et al.*, 1999; Hallgren *et al.*, 2003). In contrast, *ISEcp1* is often reported to be involved in the mobilization of resistance genes, such as CTX-M in Gram-negative bacteria, but has not been reported in Gram-positive bacteria (Poirel *et al.*, 2005; Cao *et al.*, 2002). The *aadA* gene located in a class 1 integron has also been reported in *Enterococcus faecalis* (Clark *et al.*, 1999).

The genetic environment of a novel *aph(2'')* allele [*aph(2'')-Ie*] conferring HLGR was investigated, and the surrounding sequences that included the streptomycin adenylyltransferase gene (*str*) and an *ISEcp1*-like element were described.

METHODS

Bacterial strains, plasmids, and susceptibility testing. The *Enterococcus casseliflavus* strain used in this study (HZ95) was originally isolated from the ascites of a patient at the intensive care unit (ICU) of a teaching hospital in China. The strain was identified to the species level by the API 20 Strep system and Vitek GPI (bioMérieux) for Gram-positive organisms, and was confirmed to have the *vanC2* gene by PCR amplification. Strains and plasmids used in this study are shown in Table 1. One hundred and two clinical enterococci (44 *Enterococcus faecium* isolates, 48 *Ent. faecalis* isolates, six *Enterococcus gallinarum* isolates, three *Ent. casseliflavus* isolates and one *Enterococcus avium* isolate) from unrelated patients in the hospital were used to screen for HLGR and the novel aminoglycoside-resistance genes. *Staph. aureus* ATCC 25923 and *Ent. faecalis* ATCC 29212 were used as the reference strains for quality control. MICs of seven aminoglycosides, gentamicin, netilmicin, tobramycin, kanamycin, amikacin, isepamicin and streptomycin, to *Escherichia coli* pT95 and *E. coli* pGEM-T Easy were determined by the agar dilution method. MICs of these seven aminoglycosides, ampicillin, vancomycin, teicoplanin and linezolid were also performed for the *Ent. casseliflavus* HZ95 strain. Interpretation of the results was according to Clinical and Laboratory Standards Institute [CLSI; formerly the National Committee for Clinical Laboratory Standards (NCCLS)] 2005 guidelines (CLSI, 2005).

Screening for strains displaying HLGR. HLGR (MIC > 500 µg ml⁻¹) was confirmed by an agar screening method according to CLSI (2005). The isolate was dotted at 10⁶ c.f.u. on brain heart infusion (BHI) plates containing gentamicin (500 µg ml⁻¹), and was incubated at 35 °C for 24 h. Growth of more than one colony was determined to be the best indicator of HLGR.

Detection of aminoglycoside-resistance genes by PCR. The presence of the genes for the aminoglycoside-modifying enzymes (AMEs) responsible for high-level aminoglycoside resistance was confirmed by PCR. The studied genes were *aac(6')-Ie-aph(2'')-Ia*, *aph(2'')-Ib*, *aph(2'')-Ic*, *aph(2'')-Id*, *aph(3')-IIIa*, *aac(6')-Ii*, *ant(3')-Ia*, *ant(4')-Ia* and *ant(6')-Ia*. PCR reactions were done on bacterial lysates. PCR assays were performed to amplify these genes as described previously (Donabedian *et al.*, 2003). Strains that were positive for *aac(6')-Ie-aph(2'')-Ia*, *aph(2'')-Ib*, *aph(2'')-Ic*, *aph(2'')-Id*, *aph(3')-IIIa*, *aac(6')-Ii*, *ant(3')-Ia*, *ant(4')-Ia* and *ant(6')-Ia* in our laboratory were employed as positive controls, and *Ent. faecalis* ATCC 29212 was used as a negative control. We confirmed all positive results by sequencing the PCR product, for which *Ent. faecalis* ATCC 29212 was again used as a negative control strain.

Purification, cloning and sequencing of PCR products. PCR products were purified by using the 3S Spin PCR Product Purification kit (Shenergy Biocolor). Cloning was done by standard methods, using the purified PCR products, the pGEM-T Easy vector and *E. coli* DH5α. The PCR products were sequenced by the dideoxynucleotide chain-termination method on an ABI 377 sequencer. Sequence analysis employed BLAST from the National Center for Biotechnology Information (NCBI). DNAssit version 2.2 software was used to compare similarities among other sequences.

Conjugative experiments. Conjugation experiments were carried out using a broth mating technique between the clinical isolate *Ent. casseliflavus* HZ95 (displays HLGR and is susceptible to rifampicin and fusidic acid) and *Ent. faecalis* JH2-2 (fusidic acid and rifampicin resistant and gentamicin susceptible) or *Ent. faecium* BM4105-RF (rifampicin resistant and gentamicin susceptible), as previously described, with a donor/recipient ratio of 1:2. The mating time of broth mating was 4 h (Ike *et al.*, 1998). Portions of the mixed cultures were then transferred to plates of solid media with appropriate selective antibiotics. Colonies were counted after 48 h incubation at 37 °C.

Plasmid isolation, preparation of probe, and Southern blot hybridization. A Qiagen Plasmid Midi kit was used to extract plasmids in HZ95. Plasmids were digested with the restriction enzyme *HindIII*. After agarose gel electrophoresis, DNA fragments in the gel were transferred to a nylon membrane. The DNA fragment of the *aph(2'')-Id* gene in the strain HZ95 obtained by PCR amplification (Tai *et al.*, 1998) was purified as described above. The purified fragment (642 bp) was labelled with the Prime-H-Gene ³³P probe labelling kit (Promega) for use as a DNA probe.

Cloning of the plasmid-encoded gentamicin-modifying enzyme gene. Plasmid DNA was extracted from HZ95 by the Qiagen Plasmid Midi kit and digested with *HindIII*. Purified digested product, 3.5 mmol Mg²⁺ l⁻¹, 200 µmol dNTPs l⁻¹ and 1.6 U *Taq* polymerase were added to 1 × PCR buffer. The mixture was extended in a PCR amplifier (ThermoHybaid) at 72 °C for

Table 1. Reference strains and plasmids used in this study

Strain or plasmid	Characteristics	Source or reference
Strains		
<i>E. coli</i> pGEM-T Easy	<i>E. coli</i> DH5α harbouring pGEM-T Easy vector	This study
<i>E. coli</i> pT95	Transformant, <i>E. coli</i> DH5α harbouring pT95	This study
Plasmids		
pGEM-T Easy	Ampicillin resistant, empty vector	Promega
PT95	Recombinant pGEM-T Easy plasmid with an 8724 bp <i>HindIII</i> fragment containing <i>aph(2'')-Ie</i> insert	This study

Fig. 1. The cloned *Ent. casseliflavus* fragment of four ORFs, 8724 bp in total. The fragment was cloned from plasmids of HZ95 digested by *Hind*III. The arrows indicate the orientations of the ORFs.

30 min. The cohesive terminal was repaired and adenosine deoxyriboside (A) was added to the terminal. Purified product (3 µl) was linked with 1 µl pGEM-T Easy vector. The linked product was transformed to *E. coli* DH5α competent bacteria, and then the resultant bacteria were spread on a MacConkey plate containing 50 µg ampicillin ml⁻¹ and 8 µg gentamicin ml⁻¹. White colonies designating the *E. coli* pT95 clone were picked the following day. The plasmid was extracted and digested with *Eco*RI to check for insertions in the vector DNA. The purified transformant plasmid DNA from *E. coli* pT95 clones with an insert of the target DNA was used as the template for the walking sequencing reaction (Fig. 1). A similarity search of the sequence was carried out using the BLAST program available at the NCBI BLAST homepage (<http://www.ncbi.nlm.nih.gov/blast/>).

Identification of the *aph(2'')*-*Ie* and *str* genes in enterococci.

Novel primers (P1, 5'-GGATCCGTAGAGAAGACATGAC-3'; P2, 5'-CTCGAGCATTAGCAGTCC-3') were designed to amplify the whole sequence of the *aph(2'')*-*Ie* gene, and additional primers (P3, 5'-CGCAGTTTCTGAATTTGCTTATC-3'; P4, 5'-CGCTTCTATATCCATTCATCTCA-3') were designed to amplify the *str* gene. PCR assays were performed to amplify the *aph(2'')*-*Ie* and *str* genes among the 102 clinical enterococci.

RESULTS

Microbiological characterization

Ent. casseliflavus HZ95 was high-level resistant to gentamicin, netilmicin, tobramycin, kanamycin, amikacin and isepamicin (MICs >1024 µg ml⁻¹), but was low-level resistant to streptomycin (MIC=128 µg ml⁻¹; Table 2). MICs of ampicillin, vancomycin, teicoplanin and linezolid for *Ent. casseliflavus* HZ95 were 256, 1, 1 and 2 µg ml⁻¹, respectively. Among 102 clinical strains, 68 (66.7%) isolates displaying HLGR were detected.

Novel gentamicin-resistance gene, *aph(2'')* allele [*aph(2'')*-*Ie*], and its location

A DNA fragment of 642 bp was obtained from total DNA of HZ95. The nucleotide sequence for the novel HGLR *aph(2'')* allele *aph(2'')*-*Ie* is available from GenBank under accession number AY677166. No positive PCR amplification result was seen in the isolate with specific primers for aminoglycoside-resistance genes, including *aac(6')-Ie-aph(2'')*-*Ia*, *aph(2'')*-*Ib*, *aph(2'')*-*Ic*, *aph(2'')*-*Id*, *aph(3')-IIIa*, *aac(6')-Ii*, *ant(3')-Ia*, *ant(4')-Ia* and *ant(6')-Ia*.

Southern-blot hybridization was used to determine the location of the *aph(2'')*-*Ie* gene in *E. casseliflavus* HZ95. Four kinds of plasmids were detected in *Ent. casseliflavus* HZ95, but positive hybridization signals were detected only in the 16 kb plasmid band and the 8.7 kb *Hind*III-digested fragment.

Conjugation, cloning and expression of the gentamicin-resistance gene

No transconjugants were selected by conjugation experiments. The plasmids from HZ95 digested by *Hind*III were ligated to the pGEM-T Easy vector. After transformation of the ligated products, the gentamicin-resistant transformants were selected. An *E. coli* DH5α derivative resistant to gentamicin was obtained and named *E. coli* pT95, and contained an 8.7 kb cloned fragment. Aminoglycoside MICs for *E. coli* pT95 and *E. coli* pGEM-T Easy are shown in Table 2. Repeated attempts to electroporate the native 16 kb plasmid into *Ent. faecalis* JH2-2 failed.

Table 2. Susceptibilities of *Ent. casseliflavus* HZ95, *E. coli* pT95 and *E. coli* pGEM-T Easy to aminoglycosides

Values show MIC (µg ml⁻¹).

Aminoglycoside	Strain		
	<i>Ent. casseliflavus</i> HZ95	<i>E. coli</i> pT95	<i>E. coli</i> pGEM-T Easy
Gentamicin	≥1024	24	0.125
Netilmicin	≥1024	8.0	0.5
Tobramycin	≥1024	8.0	0.25
Kanamycin	≥1024	8.0	0.25
Amikacin	≥1024	1.0	1.0
Isepamicin	≥1024	0.25	0.125
Streptomycin	128	2.0	1.0

Nucleotide sequencing of the cloned fragment

The nucleotide sequence of the cloned fragment revealed the presence of four ORFs, 8724 bp in total (GenBank accession no. AY939911) (Fig. 1). The nucleotide and deduced amino acid sequences of these ORFs were compared with the sequences in the GenBank database by the BLAST program.

The first ORF (373–2029 bp) was an insertion sequence 91 % identical to *ISEcp1*, and contained a transposase gene (*tnpA*) (561–1823 bp) with 30 bp inverted repeats (IRs) at both ends (GenBank accession no. AY455830).

The second ORF (3536–4441 bp) was the novel HLGR *aph(2'')* allele gene, *aph(2'')-Ie*, encoding a gentamicin-modifying enzyme. The deduced amino acid sequence had 93.7 % identity with the chromosome-encoded APH(2'')-Id AMEs reported by Tai *et al.* (1998), with 19 amino acid differences. Also, its amino acid sequence was 29.2, 31.1 and 27.8 % identical with the phosphotransferases encoded by *aph(2'')-Ia* (GenBank accession no. M13771), *aph(2'')-Ib* (GenBank accession no. AF207840) and *aph(2'')-Ic* (GenBank accession no. U51479), respectively.

The third ORF (7074–7982 bp) was the gene of a putative plasmid replication initiator protein (RepD) (GenBank accession no. X92946) which is involved in the introduction of a single-strand nick on the leading strand at the origin of replication and in the initiation of rolling-circle plasmid replication (Perreten *et al.*, 2001).

The last ORF (7990–8724 bp) was the truncated streptomycin adenylyltransferase gene (*str*), interrupted by the internal *Hind*III site, so that only part of the gene was on the ~8 kb fragment sequenced. The *str* gene belongs to the aminoglycoside adenylyltransferase (*aad*) family. Its amino acid sequence showed 100 % identity with that of the recently detected plasmid-mediated streptomycin adenylyltransferase gene from isolates of *Lactococcus lactis* (GenBank accession no. X92946) (Schwarz *et al.*, 2001; Projan *et al.*, 1988). Its amino acid sequence was also 80.3 and 13.9 % identical to 6'-streptomycin adenylyltransferase (*aadE*) and 3"-streptomycin adenylyltransferase (*aadA*), respectively, from *Enterococcus* (GenBank accession nos AY602212 and AF052459) (Derbise *et al.*, 1997; Ahmed & Shimamoto, 2004; Clark *et al.*, 1999).

Identification of the *aph(2'')-Ie* and *str* genes in enterococci

Among 102 clinical enterococci, one *Ent. faecium* isolate (HZ438) and one *Ent. gallinarum* isolate (HZ472) with HLGR were confirmed to contain the *aph(2'')-Ie* gene, and three *Ent. faecalis* isolates (HZ46, HZ57 and HZ179) and one *Ent. faecium* isolate (HZ438) had the *str* gene. This *Ent. faecium* isolate contained both *aph(2'')-Ie* and *str*.

DISCUSSION

The *aac(6')-Ie-aph(2'')-Ia* gene is the main AME gene associated with HLGR, encoding AAC(6')-Ie-APH(2'')-Ia.

This bifunctional enzyme can cause the resistance of the enterococcus to essentially all clinically available aminoglycosides, except streptomycin, and eliminates the synergism between aminoglycosides and a cell-wall-active agent (Ferretti *et al.*, 1986). The complete or truncated Tn5281 containing the *aac(6')-Ie-aph(2'')-Ia* gene with IS256 upstream and/or downstream has been detected worldwide among strains displaying HLGR (Simjee *et al.*, 1999; Hallgren *et al.*, 2003). This is one explanation for the higher frequency of enterococci with HLGR. APH(2'')-Ic, APH(2'')-Id and APH(2'')-Ib are comparatively rare in enterococci (Chow *et al.*, 1997; Tai *et al.*, 1998; Kao *et al.*, 2000). The *aph(2'')-Ic* gene is associated with gentamicin resistance (MIC 256–584 µg ml⁻¹) and loss of synergism (Chow *et al.*, 1997). The *aph(2'')-Id* and *aph(2'')-Ib* genes result in high-level resistance to aminoglycosides, except streptomycin (MIC ≥ 2000 µg ml⁻¹), but do not eliminate the synergism between amikacin and a cell-wall-active agent (Tai *et al.*, 1998; Kao *et al.*, 2000).

In this study, the AME APH(2'')-Ie encoded by the novel HLGR *aph(2'')* allele [*aph(2'')-Ie*] was detected, and differed from APH(2'')-Id by a 19 aa substitution. The surrounding genetic structure of the *aph(2'')-Ie* gene encoding this enzyme in *Ent. casseliflavus* HZ95 was determined in detail. The *aph(2'')-Ie* gene was confirmed to reside on a 16 kb plasmid. By comparison of the MICs of aminoglycosides for *E. coli* pT95, containing the *aph(2'')-Ie* gene, and *E. coli* pGEM-T Easy, it was shown that the MICs for gentamicin, netilmicin, tobramycin and kanamycin of *E. coli* pT95 were much higher than those of *E. coli* pGEM-T Easy. It may be concluded that the *aph(2'')-Ie* gene can lead to resistance to gentamicin, netilmicin, tobramycin and kanamycin, but not to amikacin, isepamicin and streptomycin. The only functional difference between these genes is that the isolate from which *aph(2'')-Id* was obtained had a lower level of resistance to netilmicin than *Ent. casseliflavus* containing the *aph(2'')-Ie*. We detected the *aph(2'')-Ie* gene not only in *Ent. casseliflavus* but also in *Ent. faecium* and *Ent. gallinarum* detected in screens at our hospital over the course of 1 year.

Downstream of the *aph(2'')-Ie* gene, the presence of the *str* gene may be the explanation of the low-level streptomycin resistance (MIC = 128 µg ml⁻¹). The *str* gene has been reported in *L. lactis* and was first detected in *Enterococcus*. We detected the *str* gene not only in *Ent. casseliflavus* but also in three *Ent. faecalis* isolates and one *Ent. faecium* isolate. The *Ent. faecium* isolate contained both *aph(2'')-Ie* and *str* genes.

An upstream *ISEcp1*-like element was detected with a 22 aa substitution in the transposase compared to that of *ISEcp1*. *ISEcp1* is commonly related to the transmission of resistance genes such as CTX-M-type β-lactamase genes, but has not been reported in Gram-positive bacteria (Poirel *et al.*, 2005; Cao *et al.*, 2002). Accordingly, we presume that *ISEcp1* is relevant to the transmission of *aph(2'')-Ie* and *str*. According to Donabedian *et al.* (2003), the *aph(2'')-Id* and *aph(2'')-Ib* genes, which are rarely found in human isolates, are more

prevalent in several food-producing animals, and are transmitted from food-producing animals to humans through the food supply, since gentamicin is commonly used in swine, and widely used in chickens and turkeys. It is presumed that these novel aminoglycoside-modifying genes and the IS in *Enterococcus* were acquired from the environment. The presence of these two linked aminoglycoside-resistance genes that might have been transferred from diverse bacterial species is another example of how bacteria evolve to become more resistant to a broader spectrum of antimicrobial agents.

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